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A NEW FACILE TRINITROPHENYLATED SUBSTRATE FOR PEPTIDE α-AMIDATION AND ITS USE TO CHARACTERIZE PAM ACTIVITY IN CHROMAFFIN GRANULES

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SUMMARY: Carboxyl terminal α-amidation is a prevalent post translational modification in neuropeptide hormones, with amidation being essential for biological activity. We report a direct demonstration and characterization of peptidyl α-amidating monooxygenase (PAM) activity in chromaffin granules, secretory vesicles long known as loci for synthesis and storage of catecholamines but only recently recognized as processing and storage sites for neuropeptides. This finding, together with the recently recognized competence of dopamine-b-monooxygenase to carry out N-dealkylation, provides important information regarding the co-localization and co-secretion of multiple neuromodulators. In addition, we introduce a new substrate for both pituitary and chromaffin granule PAM -- TNP-D-Tyr-Val-Gly. This substrate exhibits high turnover, and has the important advantage of allowing quantitative activity determinations using standard spectrophotometric techniques, thus facilitating mechanistic studies and inhibitor development.

Neurohormonal peptides are vitally involved in a wide variety of central and peripheral functions in mammals. Like other biologically active peptides, they usually arise via enzymatic processing of larger precursor proteins, and these post-translational modification reactions are currently the focus of intense interest in many laboratories. A striking feature of peptide neurohormones is that more than 50% of the known peptides have an α-amide group on the carboxyl terminal side. The amide group is necessary for the bioactivity, and also may provide resistance to carboxypeptidases and may be important in the regulation of peptide hormones (1,2). Amidation is catalyzed by a copper- and ascorbate- requiring monooxygenase -- peptidyl α- amidating monooxygenase (PAM) -- which has been purified from pituitary and characterized (3,4,5). It has been demonstrated that PAM catalyzes oxygenative cleavage at the C-terminal glycine of the precursor peptide to give the amidated neuropeptide and glyoxalate, but detailed mechanistic information is not yet available on this key enzymatic reaction.

Adrenal chromaffin granules have been well characterized as storage vesicles for biogenic amines (6,7), but it is only recently that they have been recognized as processing and storage sites for neuropeptides. Initially, evidence showed the co-existence and co-secretion of opiate-like peptides and catecholamines in the adrenal medulla (8), and later enkephalins were identified in adrenal medulla (9). Several enkephalin- and proenkephalin-like molecules, as well as other types of neuropeptides including

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the amidated peptides adrenorphin (10), amidorphin (11) and neuropeptide Y (12), were later identified in chromaffin granules. There exists, therefore, circumstantial evidence for the presence of an enzymatic system in chromaffin granules capable of post-translational carboxyl terminus amidation of peptides.

We now wish to report a direct demonstration that adrenal medullary chromaffin granules are indeed capable of α-amidation, and that the reaction is enzymatically catalyzed and has the oxygen and ascorbate requirements and copper stimulation that characterize pituitary PAM. We find that the amidating activity of adrenal chromaffin granules is stable and present in both the soluble and membranous fractions. In addition, we introduce TNP-D-Tyr-Val-Gly as a new, sensitive substrate for PAM-like activity. This substrate gives high turnover with both pituitary and chromaffin granule fractions, and it has the important advantage of allowing quantitative determinations of specific activities using standard spectrophotometric techniques, thus facilitating mechanistic studies and inhibitor development.

MATERIALS AND METHODS

Bovine adrenals were obtained from a local meat packing firm and the medullae were dissected within one hour post-mortem. Chromaffin granules were prepared by previously published methods (13; as modified in 14), with the addition of 5,000 U/ml catalase (Boehringer) in the final buffer, and frozen. In a typical preparation, 15mL of granules were thawed, diluted to 50mL with 20mM KPi pH 7.0, and centrifuged at 41,000g for 15min. The pellet was homogenized in another 50mL buffer and recentrifuged. The combined supernatants were subjected to ammonium sulfate fractionation and the 0-50% fraction was suspended in 3mL 100mM Na TES pH 7.0. Freezing for several weeks, and several freeze-thaw cycles did not alter the enzymatic activity. The pellet was dissolved in 5mL TES buffer containing 1% Triton X-100 (Pierce), and centrifuged at 50,000g for 30min, the supernatant contained the solubilized membranous fraction of the granules. Protein assays were performed using the Biorad reagent and bovine serum albumin as a standard.

PAM homogenates were prepared from frozen bovine pituitaries (Pel-Freeze Biologicals) as described elsewhere (15). The 25-45% ammonium sulfate pellet of dissected and separated neurointermediate lobes was suspended in 20mM Na TES, pH 7.0, and used for assays.

Assays were performed by a modification of published procedures (16,17). The UV-active trinitrophenyl (TNP) group was attached to the N-terminus of D-Tyr-Val-Gly and the resulting modified tripeptide was used as a substrate. Incubations were performed at 37 ^{0}C in a total volume of 500μL 100mM Na TES, pH 7.0, that contained: 10mM ascorbate, 2μM $CuSO_4$, 6,500 U catalase, 5nmoles TNP-D-Tyr-Val-Gly and various amounts of enzyme fractions. After 3hr, the assay mixture was quenched with 20μL of 10M TCA, centrifuged and subjected to hplc analysis.

TNP-D-Tyr-Val-Gly was synthesized by mixing 5mg D-Tyr-Val-Gly (Sigma Chemical) in 10mL 0.1M $Na_2B_4O_7$ with 30mg trinitrobenzene-sulfonic acid (Pierce). The mixture was stirred at room temperature for 40min, 5mL of acetic acid were added, the aqueous layer was saturated with NaCl and extracted three times with 10mL $CHCl_3$. The combined $CHCl_3$ extracts were dried over Na_2SO_4 and evaporated to dryness under vacuo. The residue was washed with 10mL pentane and twice with 10mL pentane/ether (1/1), and dissolved in 3mL MeOH. TNP-D-Tyr-Val-NH_2 was synthesized in a similar manner, using D-Tyr-Val-NH_2 as the starting material. TNP-D-Tyr-Val-Gly and TNP-D-Tyr-Val-NH_2 were separated on a C8 RP column at room temperature, using a solvent of 55% 0.2M ammonium acetate,pH 4.1/ 45% acetonitrile, at a flow of 1.5 mL/min, and detected by absorbance at 344nm. The extinction coefficient of $12.2x10^3$ cm^{-1} M^{-1} at 350nm, reported in the literature for TNP-Tyr at pH 9.3 (18), and determined by us to be invariant over the pH range 4.0 to 9.5, was used for determination of absolute amounts of TNP-D-Tyr-Val-Gly and TNP-D-Tyr-Val-NH_2.

RESULTS AND DISCUSSION

D-Tyr-Val-Gly was introduced as a PAM substrate by Bradbury and coworkers, who labeled the tripeptide with ^{125}I and detected product formation by measuring radioactivity (16). This substrate has the advantage of being resistant to proteases, and easily iodinated. Recognizing that the prehormone substrates of PAM do not normally posses a free amino functionality close to the nascent C-terminal amide, our strategy was to examine the reaction of PAM with the same tripeptide modified at the amino terminus with the highly UV-active trinitrophenyl group.

As shown in Fig. 1, hplc analysis of TNP-D-Tyr-Val-Gly and TNP-D-Tyr-Val-NH_2 produced two distinct, well separated peaks. The hplc peak corresponding to TNP-D-Tyr-Val-Gly was isolated and analyzed by FAB mass spectrometry. It produced a M+1 peak at m/z 549, and another peak at m/z 571 which correspondes to the mono-sodiated derivative (Figure 1.1). The hplc peak corresponding to TNP-

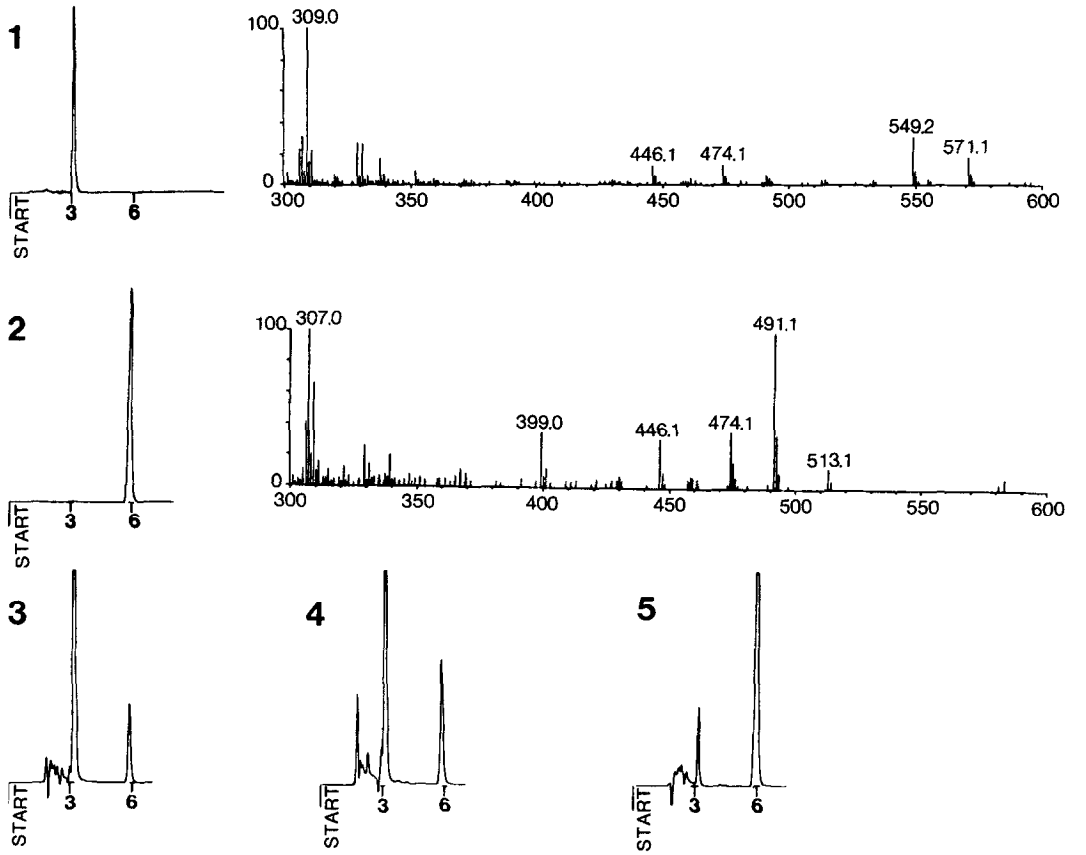

FIG 1. HPLC analyses, and mass spectra of TNP-D-Tyr-Val-Gly and TNP-D-Tyr-Val-NH_2. Analysis was performed as indicated in the Materials and Methods, except that solvent flow is at 1 ml/min; the scale on the hplc traces is in minutes. FAB mass spectra were obtained using thioglycerol as a matrix. Panels 1 and 2 are the traces and mass spectra of TNP-D-Tyr-Val-Gly and TNP-D-Tyr-Val-NH_2, respectively. Panel 3 is a 3 hour assay of 220 ng of the soluble chromaffin granule fraction. Panel 4 is the same assay, spiked with authentic TNP-D-Tyr-Val-NH_2; the peak at the void volume is caused by borate buffer present in the authentic sample. (5) is a 3 hour assay of 70 ng of the 25-45% ammonium sulfate fraction of neurointermediate pituitaries.

D-Tyr-Val-NH_2 was similarly analyzed and produced a M+1 peak at m/z 491 and a mono-sodiated peak at m/z 513 (Figure 1.2). Hplc analysis of assay mixtures produced traces such as the one shown in Figure 1.3. Authentic TNP-D-Tyr-Val-NH_2 co-eluted with the enzymatic product, as shown in the spiking experiment in Figure 1.4.

Frozen bovine pituitaries were dissected and fractionated as indicated in the Methods. The 25-45% ammonium sulfate fraction produced TNP-D-Tyr-Val-NH_2 in an enzyme dependent manner, and has the ascorbate, oxygen and copper requirements reported previously for ^{125}I-D-Tyr-Val-NH_2 formation (19). As shown in the Table, TNP-D-Tyr-Val-Gly is a highly active substrate for the pituitary PAM fraction. In our hands, the 25-45% ammonium sulfate fraction of neurointermediate pituitaries shows more than 100-fold increase in activity than the value of 1.2 nmoles/mg/hr that was previously reported with ^{125}I-D-Tyr-Val-Gly (15), although substrate may not have been fully saturating in that report. Thus, it is clear that TNP-D-Tyr-Val-Gly is indeed a facile PAM substrate, with the presence of the UV-absorbing TNP group allowing facile detection and quantitation of product formation by hplc, without resort to radiolabeling.

Bovine adrenal medullary chromaffin granules were prepared as indicated in Methods. The granules were fractionated in the soluble and membrane bound fractions and assayed for amidating activity with TNP-D-Tyr-Val-Gly. Both fractions produced TNP-D-Tyr-Val-NH_2 in a manner that exhibited all the characteristics of PAM (Table 1). As shown in Fig. 2, product formation is linear with time up to about 7 hours and exhibits both copper and ascorbate dependence. In other experiments, not shown here,

TABLE 1

COFACTOR REQUIREMENTS AND SPECIFIC ACTIVITIES OF PAM FRACTIONS

Fraction	Protein (mg/mL)	Amidating Activity[a] (nmoles/mg/h)
0-50% Amm. Sulfate	4.5	5.7
0-50% Amm. Sulfate (no asc.)	4.5	0.2
0-50% Amm. Sulfate (no O_2)	4.5	0.5
0-50% Amm. Sufate (boiled)	4.5	0.2
Membrane Fraction	2.5	6.0
Neurointermediate Pituitaries (25-45% Amm. Sulfate)	16	200

[a] Assays were performed as described in the Materials and Methods, using conditions where reaction rates were linear both in time and protein. Activity was quantified by comparison of product peak heights versus a standard curve constructed with authentic product.

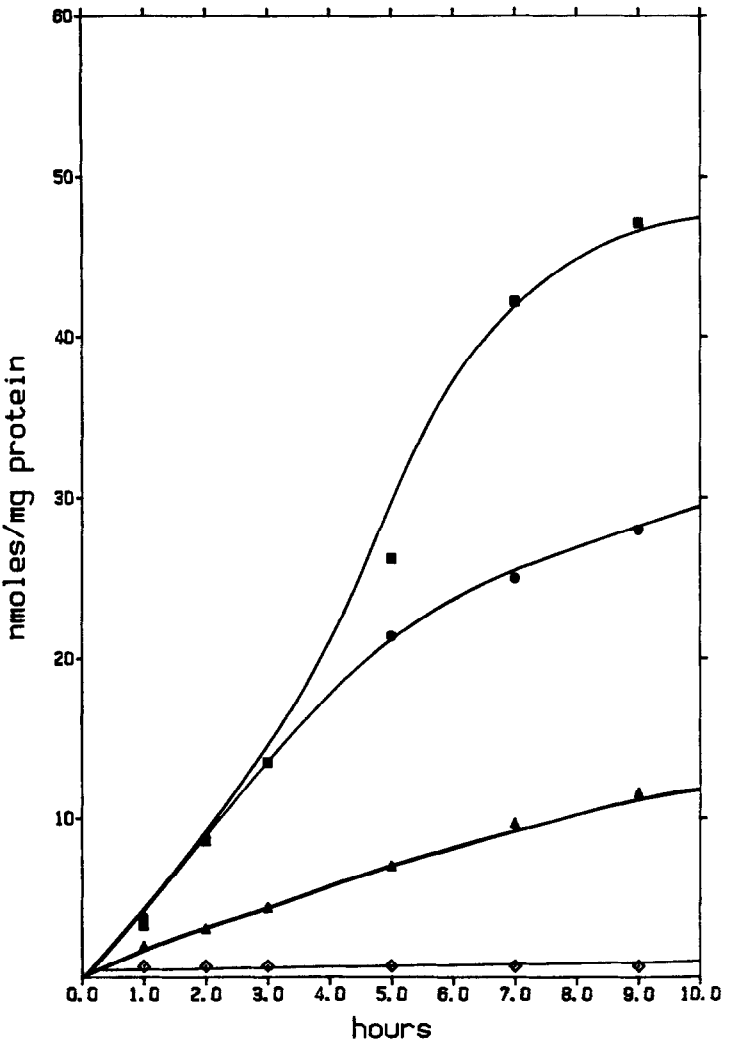

FIG. 2. Time dependent formation of TNP-D-Tyr-Val-NH_2. Assays were performed by increasing the quantities indicated in the Materials and Methods by 5-fold. Points were obtained by quenching and analyzing aliquots at the indicated times. (■) is the soluble cromaffin granular fraction, and (●) is the membrane bound chromaffin granular fraction. Controls performed were: (◈) no ascorbate, and (▲) no $CuSO_4$. When catalase was omitted from the assay mixture, a non-enzymatic rate which depended on both $CuSO_4$ and ascorbate, became evident. This is reminiscent of the non-enzymatic amidation by a copper/ascorbate system reported by Bateman et al (20).

we observed that product formation is linear almost until 100% substrate conversion.

Amidating enzymes have been reported in a variety of mammalian tissues, including the pituitary, hypothalamus, thyroid, pancreas, cerebrospinal fluid and serum (21). This report represents a direct demonstration and the first characterization of amidating activity in chromaffin granules; a recent preliminary report of PAM-like activity in chromaffin granules has also appeared (22). The finding of PAM-like activity is of particular interest, in view of the fact that dopamine β-monooxygenase (DBM), a major enzymatic component of chromaffin granules, is the only other known mammalian copper-containing and

ascorbate-requiring monooxygenase. Our laboratory has recently reported that DBM readily catalyzes oxygenative N-dealkylation and that the mechanism involves initial electron abstraction to form the nitrogen cation radical (23,24). Since the amidation reaction performed by PAM is chemically analogous to this N-dealkylation activity of DBM, a similar mechanism can be envisioned for the PAM reaction. However, a pathway involving initial attack of activated oxygen on the a-carbon -- as is apparently operative for P-450 O-dealkylation reactions but not for DBM (24) -- remains a viable alternative.

The facility with which PAM catalyzes amidation of our substrate certainly reflects the kinetic consequences of modification of the amino end of the tripeptide. The TNP-blocked amino-group is uncharged at the assay pH, and this may allow for tighter or more productive binding at the active site. In this regard, we note that PAM, has been shown to have affinity for peptides terminating in hydrophobic residues (3), and also that dansyl-D-Tyr-Val-Gly has been shown to be a PAM substrate (25). In any case, the high activity together with the ease of handling compared to the radioactive material, make TNP-D-Tyr-Val-Gly an excellent substrate for the study of PAM-like enzymes. Furthermore, the fast and accurate product quantitation possible using the hplc procedure we report here make this the substrate of choice for much-needed mechanistic and specificity studies, which could well lead to PAM-targeted inhibitors and effectors with clinical potential.

Co-localization and co-secretion of multiple neuromodulators is an area of great interest (26). While it is possible that distinct sub-populations of chromaffin granules are the loci of DBM activity on the one hand and the PAM-like activity we report here on the other hand, the similarity between the characteristics of these two enzymatic reactions is intriguing. Further studies will help elucidate the complex and little-understood inter-relationship between the processing and function of peptide hormones and catecholamine neurotransmiters.

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